# SUBCELLULAR DISTRIBUTION OF PROSTAGLANDIN AND GONADOTROPIN RECEPTORS IN BOVINE CORPORA LUTEA

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## SUMMARY

The subcellular distribution of prostaglandin (PG) $E_1$, $F_2\alpha$ and gonadotropin receptors in bovine corpora lutea was critically examined by preparing various subcellular fractions, assaying for various marker enzymes to assess the purity and examining $^3$H-$PGE_1$, $^3$H-$PGF_2\alpha$ and $^{125}$I-human lutropin (hLH) specific binding. The marker enzyme data suggested that subcellular fractions were relatively pure with little or no cross contamination. The binding of $^3$H-PGs and $^{125}$I-hLH was markedly enriched in plasma membranes with respect to homogenate. The other subcellular fractions also exhibited binding despite very little or no detectable 5'-nucleotidase activity. If 5'-nucleotidase was assumed to lack sensitivity and reliability to detect minor contamination with plasma membranes and $^3$H-PGs or $^{125}$I-hLH binding were used as sensitive plasma membrane markers, it was still difficult to explain binding in other fractions based on plasma membrane contamination. Therefore, these results lead to the inevitable conclusion that plasma membranes were primary (or one of the primary) but not exclusive sites for $PGE_1$, $PGF_2\alpha$ and gonadotropin receptors.

## INTRODUCTION

The presence and properties of prostaglandin (PG) $E_1$, $F_2\alpha$ and gonadotropin receptors in plasma membranes of bovine corpora lutea have been previously reported by our laboratory as well as others (1-12). In only two of these reports has it been critically examined whether plasma membranes were primary or exclusive sites for $PGF_2\alpha$ and gonadotropin receptors (7,10). Since such information was lacking in the case of $PGE_1$ receptors, we have undertaken the present work. During the above studies, we also looked simultaneously at the subcellular distribution of $PGF_2\alpha$ and gonadotropin receptors. The results demonstrate that PGs and gonadotropin receptors were primarily but not exclusively localized in plasma membranes of bovine corpora lutea, were unexpected.





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## MATERIALS AND METHODS

Unlabeled $PGE_1$ and $PGF_2\alpha$ were generously supplied by Dr. John Pike of the Upjohn Company. Unlabeled human lutropin (hLH) (LER-960; 4620 IU/mg) was generously donated by Dr. Leo Reichert, Jr., of Emory University. Unlabeled human choriogonadotropin (hCG) (CR119; 11,600 IU/mg) was a gift from the Center from Population Research, National Institute of Child Health and Human Development, National Institutes of Health. The following items were purchased from indicated commercial sources: $^3$H-$PGE_1$ (sp. act. 87 Ci/mmole), $^3$H-$PGF_2\alpha$ (sp. act. 179 Ci/mmole) and carrier free $^{125}$I-Na from New England Nuclear Corp.; sodium salt of AMP, NADH, NADP, NMN, ATP, nicotinamide, cytochrome C, glucose-6-phosphate and alcohol dehydrogenase from Sigma Chemical Co.; Polyethylene glycol from Union Carbide Corp.; Gelman Metricel filters (0.45 μm pore size) from Scientific Products and ultra pure sucrose from Schwarz-Mann Biochemicals.

Procedures for checking the purity of $^3$H-PGs and their purification, if needed, were the same as those described earlier for $^3$H-$PGF_2\alpha$ except the solvent system, i.e. toluene: dioxane: acetic acid (20:10:1) (6). Unlabeled hLH was iodinated by the lactoperoxidase technique essentially as described by Miyachi et al (13). The specific activity of $^{125}$I-hLH was 49.8 μci/μg. The rest of the details on iodination were the same as described before for hCG (14).

Bovine corpora lutea of pregnancy were collected in a local slaughterhouse and placed in ice cold homogenizing buffer which consisted of 10 mM Tris-HCl, pH 7.3, 250 mM sucrose and 1 mM $Ca^{2+}$. The luteal tissue was scraped off from the rest of the ovarian tissue, minced and homogenized by five up and down strokes in a glass teflon homogenizer. The homogenates were filtered through four layers of cheese cloth. After saving an aliquot of homogenate, the rest was subjected to differential and discontinuous sucrose gradient centrifugations in order to obtain various subcellular fractions (10). Great care was taken during the collection of fractions from different sucrose density interfaces to avoid cross contamination. The so called $F_1$ and $F_{11}$ plasma membrane fractions ( 10 ) were pooled. Following isolation and washing, fractions were stored in aliquots at -20° until used. Aliquots of subcellular fractions were diluted in 0.1 N sodium hydroxide containing 0.1% sodium dodecyl sulphate and heated for 5 min at 80°. An aliquot of this digest was then taken for protein determination according to Lowry et al (15) using bovine serum albumin as the standard. The inorganic phosphate (in 5'-nucleotidase assay) was measured according to Fiske and Subbarow (16) using $KH_2PO_4$ as the standard.

The procedural details in marker enzyme assays are the same as those described earlier: Cytochrome C oxidase according to Cooperstein and Lazarow (17); glucose-6-phosphate dehydrogenase according to Kelly et al (18); NAD pyrophosphorylase according to Kornberg (19); NADH cytochrome C reductase according to Mahler (20) and 5'-nucleotidase according to Emmelot and Bos (21). The above marker enzyme assay conditions were, however, standardized with respect to assay buffer, time and the amount of subcellular fractions protein to use so that linear reaction rates were obtained. The enzyme specific activities were calculated from linear rate data.

The binding studies were conducted at 38° for 1 hr for $^3$H-$PGE_1$, 22° for 2 hrs for $^3$H-$PGF_2\alpha$ and at 38°for 2 hrs for $^{125}$I-hLH. About 0.1 μCi of $^3$H-PGs were used per tube in the binding studies. Because of the differences in specific activities, the concentrations were different for each prostaglandin, i.e. $^3$H-$PGE_1$~ 10 nM; $^3$H-$PGF_2\alpha$ ~ 5 nM. The concentration of $^{125}$I-hLH used in the binding studies was $1.0 \times 10^{-10}$M. The separation of bound and free $^3$H-PGs and $^{125}$I-hLH for all subcellular fractions other than cytosol was accomplished by filtration through Metricel filters, the details of which are the same as those described earlier (3, 5, 22). The separation of bound and free, in the

case of cytosol, was done by charcoal absorption of free $^{3}$H-PGs (23) and double precipitation of bound $^{125}$I-hLH with 10.5% polyethylene glycol (24).

Nonspecific binding was determined in each experiment using the same amount of protein, $^{3}$H-PGs and $^{125}$I-hLH as were used in the total binding tubes but in the presence of excess corresponding unlabeled ligands (PGs - 2.8 x $10^{-5}$M; hCG 1.3 x $10^{-7}$M). We could not use unlabeled hLH to assess $^{125}$I-hLH nonspecific binding because of lack of sufficient hormone. Furthermore, use of unlabeled hCG for this purpose appears to be valid primarily because $^{125}$I-hLH appears to bind to the same sites as $^{125}$I-hCG but with lower affinity (25). Nonspecific binding was essentially the same as binding to the filters (blanks) in the absence of subcellular fractions. However, there was one exception, i.e. $^{125}$I-hLH nonspecific binding in nuclei was higher than in other fractions. The significance of this observation is not known. The specific binding was the difference between total and nonspecific binding. The fmoles bound were calculated using molecular weights of 354 for $^{3}$H-PGs and 30,000 for $^{125}$I-hLH.

The marker enzyme assays and binding experiments were done simultaneously and were completed within a week after preparation of the fractions. The enzyme activities and bindings were determined using two different protein concentrations and in quadruplicates (binding). Each experimental value represents the mean and its standard error of 4 to 16 independent observations in two different experiments.

## RESULTS AND DISCUSSION

Table I shows 22 fold enrichment (with respect to homogenate) of NAD pyrophosphorylase in nuclei; 17.9 fold enrichment of cytochrome C oxidase in mitochondria; 31.9 fold enrichment of 5'-nucleotidase in plasma membranes and 90.0 fold enrichment of NADH cytochrome C reductase in microsomes. Glucose 6-phosphate dehydrogenase showed essentially no enrichment, nevertheless, it was undetectable in any fraction other than cytosol. This lack of enrichment was because the cytosol was not purified unlike other fractions. Although the above marker enzymes were enriched in appropriate fractions, they were either undetectable (NAD pyrophosphorylase and cytochrome C oxidase) or detectable only to a minor extent (5'-nucleotidase and NADH cytochrome C reductase) in the other subcellular fractions. The low detection of the latter two enzymes in other subcellular fractions may represent minor contamination and/or intrinsic activity in these fractions. Nonetheless, the above data suggests that marker enzyme distribution in bovine corpus luteum was very similar to a variety of other tissues and that the subcellular fractions isolated were relatively pure with little or no cross contamination.

When all the above subcellular fractions were tested for binding with

Table 1. Marker Enzyme Distribution in Subcellular Fractions of Bovine Corpus Luteum

| Marker Enzyme | Homogenate | Nuclei | Mitochondria | Plasma Membranes | Microsomes | Cytosol |
|---|---|---|---|---|---|---|
| NAD Pyrophosphorylase (pmoles NAD formed/min/ mg protein) | 0.7 ± 0.2 | 15.4 ± 2.9 | ND | ND | ND | ND |
| Cytochrome C Oxidase (pmoles oxidized/min/ mg protein) | 16.2 ± 1.8 | ND | 289.8 ± 15.4 | ND | ND | ND |
| 5'-Nucleotidase (nmoles Pi released/ min/mg protein) | 18.3 ± 1.2 | ND | 5.8 ± 3.1 | 584.0 ± 9.9 | 3.9 ± 2.8 | ND |
| NADH Cytochrome C reductase (pmoles reduced/min/mg protein) | 9.9 ± 1.1 | ND | 1.4 ± 0.8 | ND | 899.7 ± 56.2 | ND |
| Glucose-6-phosphate dehydrogenase (nmoles NADPH formed/ min/mg protein | 289.9 ± 15.8 | ND | ND | ND | ND | 331.0 ± 11.6 |

ND denotes non-detectable

Table 2. The Specific Binding of $^{3}$H-PGs and $^{125}$I-hLH to Subcellular Fractions of Bovine Corpus Luteum.

| Fraction | $^{3}$H-$PGE_1$ | $^{3}$H-$PGF_2\alpha$ | $^{125}$I-hLH |
|---|---|---|---|
| | Bound (fmoles/mg protein) | | |
| Homogenate | 15.3 ± 2.2 | 21.6 ± 1.6 | 7.0 ± 0.3 |
| Nuclei | 24.0 ± 6.6 | 4.4 ± 0.5 | 5.6 ± 1.0 |
| Mitochondria | 46.7 ± 8.0 | 32.4 ± 4.4 | 14.7 ± 2.0 |
| Plasma Membranes | 223.9 ± 21.1 | 192.5 ± 12.1 | 54.9 ± 4.1 |
| Microsomes | 17.6 ± 7.0 | 18.9 ± 2.4 | 1.5 ± 0.8 |
| Cytosol | 9.7 ± 3.3 | 5.1 ± 1.1 | 7.0 ± 1.5 |

$^{3}$H-$PGE_1$, $^{3}$H-$PGF_2\alpha$ and $^{125}$I-hLH, binding was markedly enriched (14.6 fold for $PGE_1$; 8.9 fold for $PGF_2\alpha$ and 7.8 fold for $^{125}$I-hLH, with respect to homogenate) in plasma membranes (Table 2). All the other subcellular fractions also exhibited binding, some of which could even be considered slightly enriched. The binding in other subcellular fractions was unexpected because the 5'-nucleotidase activity was either undetectable or detectable only to a minor extent in these fractions (see Table 1). Therefore, the possibility of plasma membrane contamination being responsible for observed binding in these other fractions appears unlikely. Before being convinced of the above statement, we entertained the thought that perhaps 5'-nucleotidase activity may lack the sensitivity and reliability needed for the detection of minor contamination with plasma membranes. If this were true, $^{3}$H-PGs or $^{125}$I-hLH binding could be considered as a sensitive plasma membrane marker (note that binding was in fmoles as compared to nmoles for 5'-nucleotidase) in other subcellular fractions. This is because of the implicit assumption in the above thought that plasma membranes may indeed be exclusive sites for PG and gonadotropin receptors. Since plasma membrane con-

Table 3. Ratio of $^{3}$H-PGs and $^{125}$I-hLH Binding in Subcellular Fractions of Bovine Corpus Luteum.

| Fraction | $^{3}$H-PGE$_1$ | $^{3}$H-PGF$_{2\alpha}$ | $^{125}$I-hLH |
|---|---|---|---|
| | (Percent binding ratio)[a] | | |
| Nuclei | 10.7 | 2.3 | 10.2 |
| Mitochondria | 20.9 | 16.8 | 26.8 |
| Microsomes | 7.9 | 9.8 | 2.7 |
| Cytosol | 4.3 | 2.6 | 12.8 |

[a] $\frac{\text{fmoles/mg protein bound in a fraction}}{\text{fmoles/mg protein bound in plasma membrane}}$ X 100

The value for plasma membrane was 100% for all three ligands.

tamination should be reflected in binding, the ratio of binding in a fraction and in plasma membrane should be the same, regardless of labeled ligand used.

It can be seen from Table 3 that the binding ratios were not the same among PGs and hLH. The ratios varied considerably with ligand in all the fractions. Similar discrepencies appeared even when the data was expressed as a ratio using homogenate binding in the denominator or as a ratio of labeled ligand binding for each fraction. The above findings, therefore, lead to the inevitable suggestion that binding observed in other subcellular fractions was intrinsic and could not be attributable to plasma membrane contamination. The above suggestion was further supported by our recent findings that lysosomes and golgi vesicles prepared from mitochondria and microsomal fractions respectively, exhibited little (lysosomes) or no (golgi vesicles) 5'-nucleotidase activity and yet bound $^{3}$H-PGs and $^{125}$I-hLH as much as or higher than plasma membranes (26). In view of the above observations, it should be convincingly clear that PG and gonadotropin receptors were present in subcellular fractions in addition to

plasma membranes. Although the above data demonstrate the presence of intracellular PG and gonadotropin receptors, we, at the present time, have neither evidence for hormone entry (relevent in the case of gonadotropins and $PGF_2\alpha$) into bovine luteal cells to bind to these sites under physiological conditions nor the data to support that these sites were indeed physiological receptors and not just binding sites (It is possible that multiple intracellular loci of receptors may simply represent sequences in the biosynthetic cycle of the receptors). Such studies including the ones on examining whether these sites are identical to those in plasma membranes with respect to binding and molecular properties are currently in progress in our laboratory. It should be recalled, however, that virtually all the studies of this type simply rely on specific binding as a means to assess the subcellular distribution of receptors.

Finally, our findings on the presence of intracellular receptors for $PGF_2\alpha$ and gonadotropins are in contrast to earlier reports (7, 10) which suggest the exclusive localization in plasma membranes. In these studies, binding observed in other subcellular fractions was simply attributed to plasma membrane contamination without rigorously exploring the alternatives (7, 10).

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